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Evaluation of the Release of Mutagens from Diesel Particles in the Presence of Physiological Fluids

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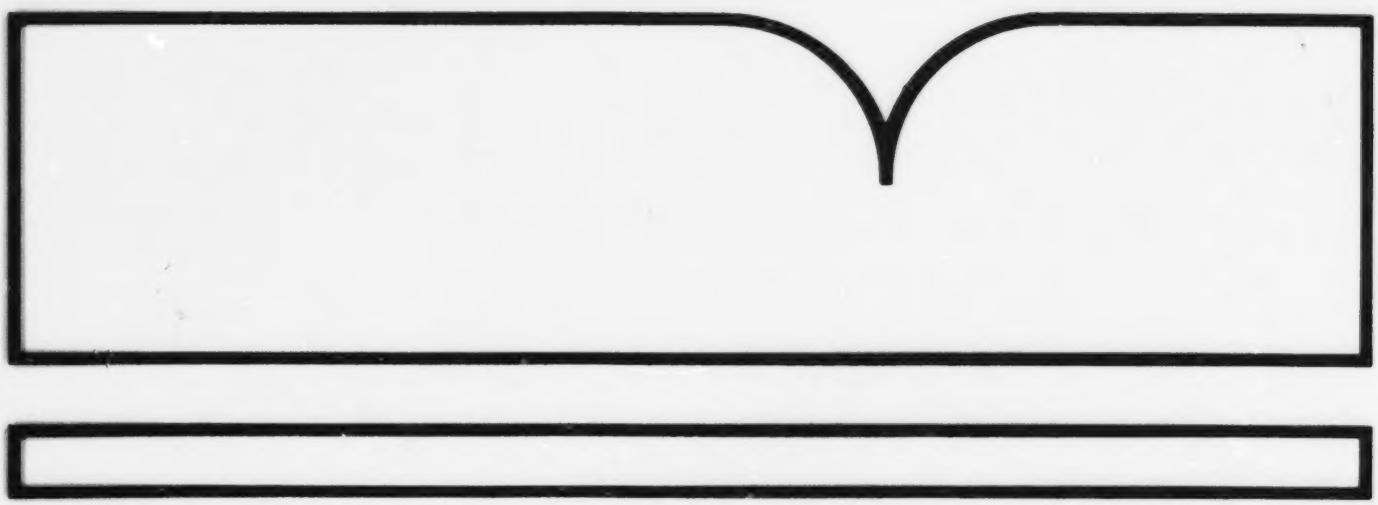

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16. ABSTRACT

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Evaluation of the Release of Mutagens From Diesel Particles in the Presence of Physiological Fluids

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The Ames Salmonella typhimurium plate incorporation assay was used to evaluate the mutagenicity of organics extracted from diesel exhaust particles. Organic solvents were more efficient than physiological fluids in removing mutagens from diesel particles, with dichloromethane extracts having the greatest mutagenic activity of the solvents examined. Serum and lung cytosol were more effective than acellular lung lavage fluid in releasing mutagenic activity from diesel particles. The mutagenic activity of diesel particle organics preextracted with dichloromethane is greatly reduced upon the addition of serum and lung cytosol to organics. Subsequently, incubation of protease with serum and lung cytosol-bound diesel organics increases the mutagenic activity. Fluorescence intensity was quantitated and found to correlate with mutagenic activity in the organic and serum extracts, but not the lung cytosol extracts. These studies suggest that substantial mutagenic activity is released from diesel particles upon incubation with serum and lung cytosol.

Key words: organic extracts, physiological extracts, mutagenicity, serum-bound mutagens, fluorescence, diesel particles

INTRODUCTION

Diesel particles have been shown to contain organic components which are mutagenic in short term bioassays [Huisingh et al, 1978]. Organic solvents are now routinely used to remove these mutagens from particles. Although studies are currently in progress to evaluate the carcinogenicity of these organics, questions still remain as to whether these potentially hazardous components are released under physiological conditions. The objective of this study was to evaluate the release of mutagens from diesel particles in the presence of organic solvents, lung fluids, lung cytosol, and human serum.

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MATERIALS AND METHODS

Human serum was purchased from Gibco Laboratories, Grand Island, NY. Protease Type V was supplied by Sigma Chemical Co., St. Louis, MO. Acetone (AC), dichloromethane (DCM), methanol (MEOH), cyclohexane (CH), dimethylsulfoxide (DMSO) and toluene (T) were of spectrograde quality and purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI.

Male New Zealand white rabbits (Charles River Breeding Laboratories, Wilmington, MA), weighing between 1 kg and 2 kg were used as the source of lung fluid. The procedure used in procuring the lung fluid by lavaging the lungs with saline was the technique of Gardner et al [1971].

Male adult rats (Charles River Breeding Laboratories, Wilmington, MA), weighing between 250 and 300 gm were used as a source for lung cytosol. The procedure used in procuring the lung cytosol, a soluble supernatant of homogenized lung tissue, was the technique of Toft and Spelsberg [1974].

Diesel particles (DP) for this study were generated and collected from a diesel passenger car, Datsun Nissan 220C, 4 cylinder, operated on a chassis dynamometer on repeated highway fuel economy cycles (10.24 miles, avg 48 mph, 12.75 minutes) by the procedure of Killough and Watson [1979]. The total exhaust was diluted and particles collected on 20in × 20in Pallflex T filters. The agglomerated particles brushed from the filters for this study had an average particle size of 2 μm.

Diesel particles were suspended at a concentration of 20 mg of particle/ml in one of the three organic solvent systems: (1) dichloromethane (DCM), (2) a mixed solvent system of 1:1:1 toluene:dichloromethane:methanol (T:DCM:MEOH); and (3) a sequential solvent system of cyclohexane followed by methanol (CH/MEOH). Particles were also suspended in human serum, lung lavage fluid, and lung cytosol at a concentration of 100 mg particles/ml.

Each particle mixture was placed into a 50-ml screw-capped glass tube (Kimble Co., Toledo, OH) and sonicated (450 watts) for 1 hour at 37°C. After sonication, the particle mixtures were centrifuged at 35,000 × g to sediment the particles. The organic solvent supernatants were evaporated to dryness at 60°C under a stream of nitrogen and reconstituted with DMSO. Previous studies in this laboratory (Zweidinger and Claxton, personal communications) have been unable to detect any loss of volatile particle-bound mutagens by this procedure when compared to solvent exchanging without evaporating to dryness. The serum, lung lavage fluid supernatants, lung cytosol, and the reconstituted organics were then passed through a 0.22-μm teflon filter (Millipore Corporation, Bedford, MA) before bioassaying to remove the remaining particles and contaminants. Organic solvents and physiological fluids controls without particles, were treated as described above and included in all bioassays. The physiological fluids were assayed for histidine content, which was found to be 0.1 mM in serum and 0.02 mM in lung cytosol. Bioassay of the fluids alone showed no mutagenic activity.

Studies to determine the optimum time for the extraction of mutagens from diesel particles were performed. Diesel particles were suspended in equal aliquots of sterile filtered serum at a concentration of 100 mg/ml and incubated in a shaking incubator (100 rpm) (New Brunswick Scientific, New Brunswick, NJ) at a temperature of 37°C and removed after incubating 1, 2, 3, and 7 days. Day 0 incubate was taken from the incubator as soon as the temperature reached 37°C (10 minutes). The particles were sedimented by centrifugation as previously described, the serum removed, and the particles washed with

200 ml of deionized water and centrifuged again at 35,000 × g for 20 minutes. After centrifugation, the water was decanted and the washed particles extracted with DCM. The DCM extract was evaporated to dryness as described previously and reconstituted with DMSO. The serum extract and the DCM extract of water-washed particles were sterile-filtered before bioassaying.

The effect of serum and lung cytosol on diesel organics added directly was investigated. Diesel particles were suspended in DCM at a concentration of 200 mg/ml in 50-ml glass tubes (screw-capped) and treated as previously described. After sonication and sedimentation of the particles, the DCM extract was evaporated to dryness and the organic residue (20 mg) was reconstituted with 10 ml of either DMSO, serum, or lung cytosol and sterile filtered.

XAD-2 ultrapure resin (40–50 mesh size) (Brinkman Instrument, Inc., Westbury, NY) was prepared using the method of Yamasaki and Ames [1977]. The technique used to chromatograph the serum extracts of particles was the method of Junk et al [1974]; however, samples were evaporated to dryness in 50-ml glass tubes.

Protease was reconstituted in deionized water at a concentration of 30 mg/ml and sterile filtered. One milliliter of the protease solution was placed in a 10-ml aliquot of sterile-filtered physiological fluids (controls and extracts of diesel particles) generated by the experiment and incubated for 30 minutes at 37°C before bioassaying.

Fluorescence spectra data were obtained by using a Perkin Elmer MPF 2A Fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan). Organic extracts of particles (0.5 ml) were diluted with DMSO to yield a total volume of 3 ml, which was placed in a quartz cuvette, and an excitation and emission spectral scan were performed. The serum and lung cytosol extracts were reextracted twice with acetone (1 ml of serum or lung cytosol extract and 3 ml of acetone). The samples were then stirred vigorously for 3 minutes and centrifuged at 500 × g for 15 minutes. Excitation and emission scans were then performed after diluting with DMSO as described above for the organic extracts of diesel particles.

In order to evaluate the correlation of mutagenic activity with fluorescence intensity, the peak height at 380 nm of the fluorescence curve was plotted versus the revertants per plate at 0.01, 0.03, 0.05, and 0.10 ml. These doses (volumes listed) are within the linear portion of the mutagenicity dose response. The correlation coefficient was determined by linear regression analysis. This analysis was applied to the following: DCM extract, T:DCM:MEOH extract, sequential solvent system of cyclohexane followed by methanol extract, and serum extract of diesel particles.

The Ames Salmonella typhimurium plate incorporation procedure with strain TA98 was used in this study and is described in detail by Ames et al [1975]. Each bioassay was performed with triplicate plates at each dose. Routinely 30–40 colonies per plate were replated on histidine-free Vogel Bonner minimal Eagles plates to determine if they were true revertants. Strains TA98 and TA100 are more sensitive to mutagenic agents due to the addition of plasmids and respond to chemicals which act either as a base pair substitution mutagen or a frame shift mutagen. Based on positive responses with TA1537 and TA1538, as well as TA98 and TA100, it appears that the active components of diesel particulate are primarily direct-acting frame shift mutagens [Huisingh et al, 1978]. The diesel sample employed in this study has been tested with all five tester strains with and without activation [Claxton and Huisingh, 1980]. The tester strain selected for the studies reported here was TA98 without microsomal activation. The organic mutagens associated with diesel particles are direct-acting and in most cases show some decrease in mutagenic activity

with the addition of the microsomal S-9 activation system. The Datsun Nissan organics used in these studies showed the most activity with strain TA98 in the absence of microsomal activation.

RESULTS

The comparative solvent study clearly indicates that organic solvents are more efficient than the physiological fluids used in removing mutagens from diesel particles (Fig. 1). DCM-extracted organics showed the greatest mutagenic activity ($P = 0.05$). Serum extract of particles (1-hour sonicate) contained mutagenic activity, but no activity was found in the lung lavage fluid extract of particles (1-hour sonicate) ($P = 0.05$). The optimum length of time for the extraction of direct-acting mutagens from particles in the presence of serum was 2 and 3 days (Fig. 2). There was a decrease in mutagenic activity after 3 days ($P = 0.05$).

In order to evaluate the release of mutagens from diesel particles in the presence of serum and other biological fluids, it was necessary to determine the direct effect of biological fluids on these mutagens in the absence of particles. Studies were then conducted with the complex organics previously extracted with DCM. The organic extracts were mixed with serum and lung cytosol prior to bioassay. The mutagenic activity was significantly reduced with both fluids (Figs. 3 and 4). When these mixtures of diesel extract

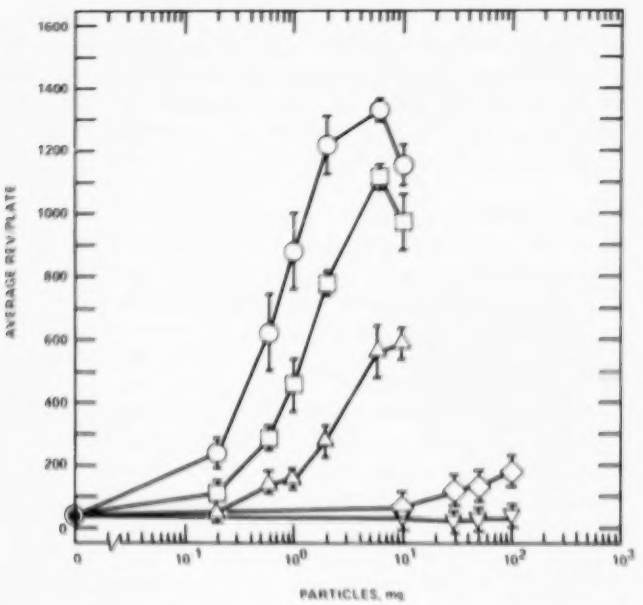


Fig. 1. Comparison of mutagenic activity detected in organic solvent extracts of diesel particles with serum and lung fluid extracts of particles after sonication using strain TA98 of Salmonella typhimurium. Bars represent standard deviation of triplicate determinations for two experiments. Symbols are as follows: ○ DCM; □ T:DCM:MEOH (1:1:1); △ Sequential CH/MEOH; ◇ Human serum; and ▽ Lung lavage fluid. One-way analysis of variance indicates a significant difference between the DCM extract and T:DCM:MEOH at $P = 0.05$.

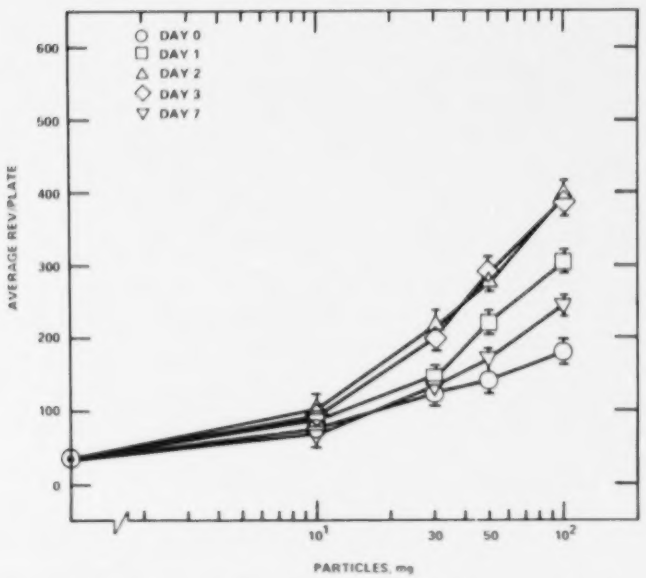


Fig. 2. Mutagenic activity detected in serum extracts of diesel particles at varying incubation times. Bars represent standard deviation of triplicate determinations for two experiments. One-way analysis of variance indicates a significant difference at $P = 0.05$.

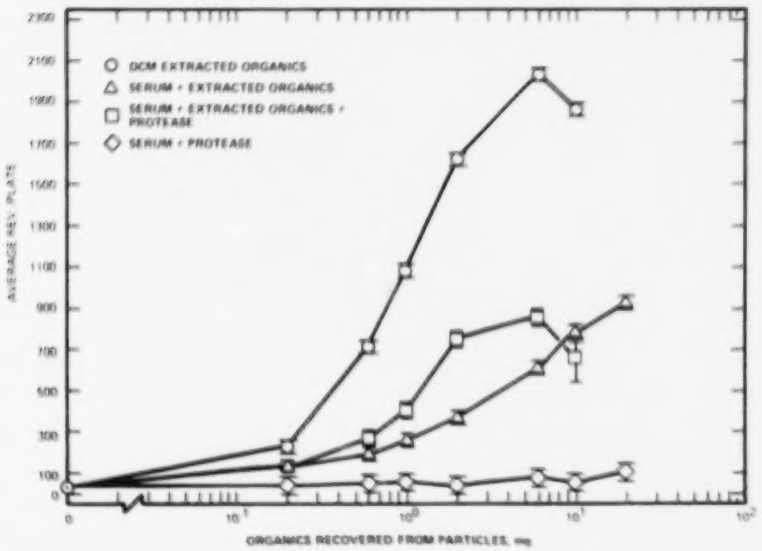


Fig. 3. The effect of serum on the mutagenic activity of diesel organics added directly. Bars represent the standard deviation of triplicate determination for two experiments.

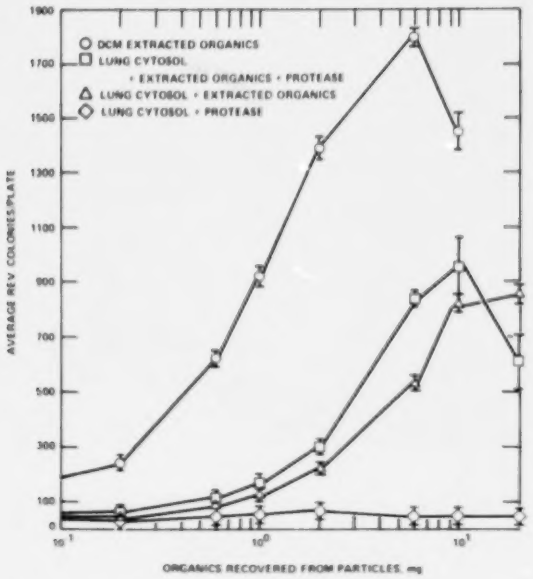


Fig. 4. The effect of lung cytosol on diesel organics added directly. Bars represent standard deviation of triplicate determination for two experiments.

plus serum or lung cytosol were incubated with protease, the mutagenic activity was slightly but significantly increased (Tables I and II). This increased activity was still below that observed for the extract alone. Lung cytosol caused a greater loss (90%) of mutagenic activity than serum (80%). Protease incubation with these mixtures resulted in a greater recovery of mutagenic activity in the case of serum (23%) compared to lung cytosol (8%), as shown in Tables I and II.

After incubation of diesel particles with serum for 2 days, the serum showed only 0.6% of the extractable mutagenic activity (Table I).

In the case of lung cytosol, no activity was detected in the cytosol after incubation with diesel particles (Table II). The particles from these 2-day incubates were carefully recovered, washed, and extracted with DCM. Bioassay of these extracts indicate that both lung cytosol and serum removed or released substantial mutagenic activity from the particles. No mutagenic activity was detected in the water wash of the diesel particle incubates. In the case of serum, 79% of the extractable mutagenic activity was removed from the particles by incubation with serum. Lung cytosol removed 85% of the mutagenic activity from particles. Control incubates of particles in the absence of either serum or cytosol showed only slight loss (6%) of mutagenic activity after washing by the same procedure followed above.

The mutagenic activity released from the particles by serum or cytosol (as determined by extracting the particle) was not observed by bioassaying the biological fluids themselves nor after the addition of protease (Tables I and II). Since initial experiments

TABLE I. Recovery of Mutagenic Activity From Diesel Particles With Serum

Sample	Slope (-MA) Rev/mg (particles) ± SD*	% of DCM extractable activity
DCM extract of diesel particles	837.6 ± 35.0[a]	100.0
DCM extract of diesel particles added to serum	169.1 ± 18.5[b]	20.0
DCM extract of diesel particles added to serum and incubated with protease (30 mg)	361 ± 11.3[b]	43.0
Serum extract of diesel particles (2 days incubation)	4.9 ± 0.1[b]	0.6
Serum extract of diesel particles (2 days incubation) incubated with protease (30 mg)	5.6 ± 0.2[b]	0.7
DCM extract of control water-washed diesel particles (in the absence of serum)	787.5 ± 0.8[b]	94.0
Water wash of diesel particles after 2 days incubation with serum	NAD[a,c]	–
DCM extract of water-washed diesel particles after 2 days incubation with serum	175.0 ± 11.3[a]	21.0

*Slope: Slope based on regression analysis (r^2 values for all samples were not less than 0.95).

[a]Mean ± SD for three separate experiments (replicate extractions and bioassay).

[b]Mean ± SD for two separate experiments (replicate extractions and bioassay).

[c]No mutagenic activity detected.

described above showed that serum added directly to extracts reduced the observed mutagenic activity, efforts were made to separate the mutagens from the serum using an XAD-2 resin.

When serum from particle incubates (serum extract) was treated with XAD-2 resin (Table III), 35% of the activity observed in serum was bound by the resin and extracted with acetone. The total resin-bound and unbound activity was 98% of that applied to the resin. After incubation of the serum extract with protease, 110% of the serum extract activity was bound by the resin and 9% eluted. Although protease significantly shifted the recoverable activity into the XAD-2-bound fraction, neither treatment increased the mutagenic activity above that observed in the serum extract itself.

Excitation and emission fluorescence spectroscopy were performed on all solvent and physiological fluid extracts from diesel particles. The maximum excitation and emission wavelengths as shown in Figure 5 A-C were as follows: (A) organic solvent extracts; excitation, 340 nm; emission, 380 nm; (B) serum extract; excitation, 340 nm; emission, 423 nm; (C) lung cytosol extract; excitation 340 nm; emission 440 nm. The maximum excitation and emission wavelength of the DCM extract of washed particles after incubation with serum was the same as the organic solvent extracts of untreated particles. The maximum excitation wavelength was 340 nm in all solvent systems examined. However,

TABLE II. Recovery of Mutagenic Activity From Diesel Particles With Lung Cytosol

Sample	Slope (-MA) Rev/mg (particles) ± SD*	% of DCM extractable activity
DCM extract of diesel particles	851.0 ± 68.9[a]	100.0
DCM extract of diesel particles added to lung cytosol	81.7 ± 3.8[b]	10.0
DCM extract of diesel particles added to lung cytosol and incubated with protease (30 mg) for 30 minutes	149.3 ± 12.5[b]	18.0
Lung cytosol extract of diesel particles (2 days incubation)	NAD[b,c]	–
Lung cytosol extract of diesel particles (2 days incubation) incubated with protease (30 mg) for 30 minutes	NAD[b,c]	–
DCM extract of control water-washed diesel particles (in the absence of lung cytosol)	787.5 ± 0.8[b]	94.0
Water wash of diesel particles after incubating with lung cytosol for 2 days	NAD[a,c]	–
DCM extract of water-washed diesel particles after 2 days incubation with lung cytosol	128.3 ± 5.66[a]	15.0

*Slope: Based on regression analysis (r^2 values for all samples were not less than 0.95).

[a]Mean ± SD for three separate experiments (replicate extractions and bioassay).

[b]Mean ± SD for two separate experiments (replicate extractions and bioassay).

[c]No mutagenic activity detected.

the excitation spectra of the organic solvent extracts (Fig. 5A) also contained fluorescence at excitation wavelengths below 310 nm which are not present in the serum and cytosol extract. The maximum emission wavelength varied from 380 nm to 440 nm as shown in Figure 5.

Since Zweidinger et al [in press] (Ann Arbor Symposium, 1978 SAE) have shown that the most mutagenically active fraction of diesel organics is highly fluorescent, quantitative fluorescence scans were performed on all the studies reported here. The intensity of the emission spectra at the maximum wavelength was determined and compared to the mutagenic activity measured by the Salmonella typhimurium bioassay. The fluorescence intensity was found to highly correlate with the mutagenic activity in all of the experiments with organic and serum extracts, as shown in Figure 6. The lung cytosol extract, although showing fluorescence (Fig. 5C), showed no measurable mutagenic activity in Salmonella typhimurium.

TABLE III. Recovery of Mutagenic Activity From Serum Extract of Diesel Particles Using XAD-2 Resin

Sample	Slope (-MA) Rev/mg (particles) ± SD*	% of Activity applied to resin
Serum extract of diesel particles	4.9	–
(XAD-2) Water eluted (bound)[a] serum extract of diesel particles	2.1[b]	43.0
(XAD-2) Acetone eluted (unbound)[c] eluted organics of serum extract of diesel particles	1.7[b]	35.0
Serum extract of diesel particles with protease	5.6	–
(XAD-2) Water eluted (bound) serum extract of diesel particles incubated with protease (30 mg)	0.5[b]	9.0
(XAD-2) Acetone eluted (unbound) organics of serum extract of diesel particles incubated with protease (30 mg)	6.2[b]	110.0

*Slope: Slope based on regression analysis (r^2 values for all samples were not less than 0.95).

[a]Bound: Mutagenic activity in the serum eluant of XAD-2.

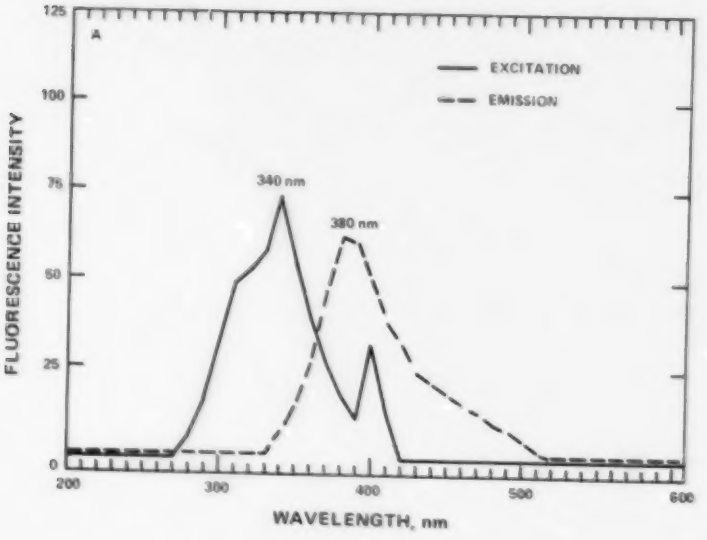
[b]One experiment (all points run in triplicate).

[c]Unbound: Mutagenic activity in the acetone eluant of XAD-2.

DISCUSSION

Diesel exhaust, as well as other fossil fuel combustion products, are known to contain carcinogens such as benzo(a)pyrene, among many other potentially hazardous and less well-characterized components [Falk and Steiner, 1952; Waller, 1952; Kotin et al, 1954; Huisingh et al, 1978]. The mutagens present in diesel emissions are frame shift mutagens, characteristic of polycyclic aromatic hydrocarbons (PAHs); however, they do not require mammalian microsomal activation. The diesel mutagens are more polar than pure PAHs and are thought to be substituted polycyclic aromatic compounds [Huisingh et al, 1978]. Numerous studies have shown that PAHs, both carcinogenic and noncarcinogenic, bind and can form stable complexes with DNA, RNA, protein of cells, cytosol protein of skin, liver, lung, and kidney. This binding is generally considered to be of primary importance for the biological phenomena of mutagenesis and carcinogenesis [Kotin et al, 1954; Heidelberger, 1964; Hey-Ferguson and Bresnick, 1971; Slaga et al, 1973; Corbett and Nettesheim, 1974; Toft and Spelsberg, 1974]. Recent studies indicate that the organics associated with diesel exhaust bind to the DNA of human cells [McCormick et al, in press].

Diesel particles are composed of a core of carbonaceous soot, very similar to carbon black. This core is coated with a complex mixture of incomplete combustion products as a result of the cooling and condensation of higher molecular weight constituents out of the gaseous emission stream. Although we have previously reported that these organics are



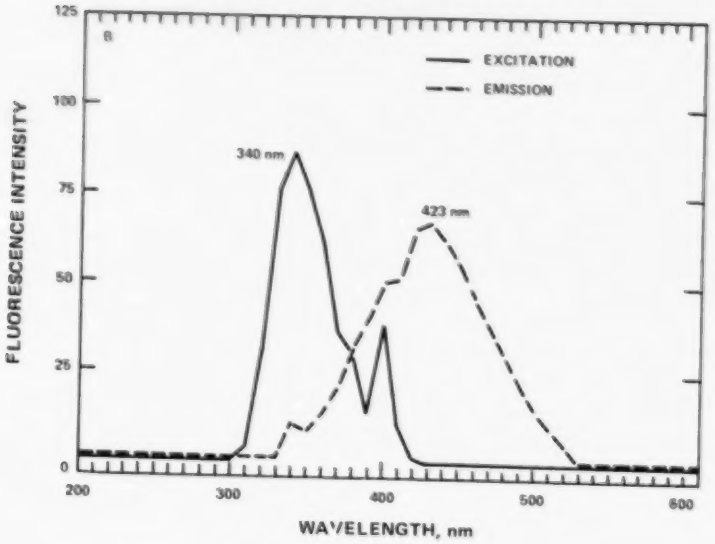


Fig. 5. Excitation (—) and emission (– –) spectra of volume-normalized extracts of diesel particles (concentrations not determined). (A) Organic solvent extract of diesel particles, (B) serum extract of diesel particles, and (C) lung cytosol extract of diesel particles, performed as described in the Methods section.

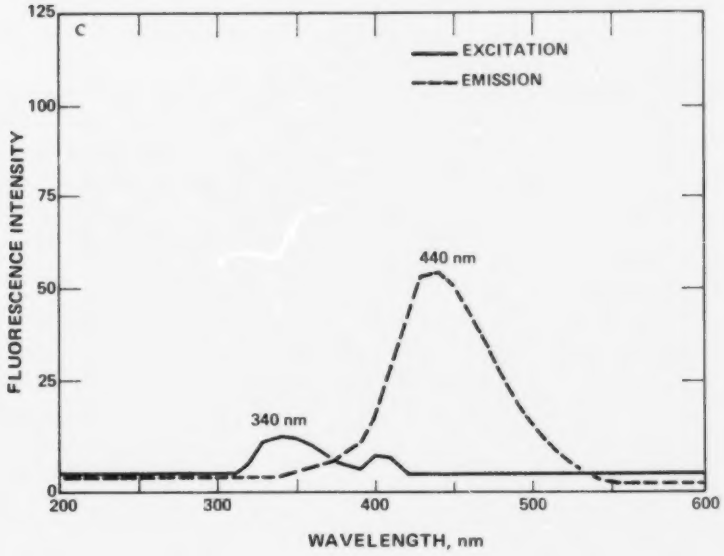


Fig. 5C.

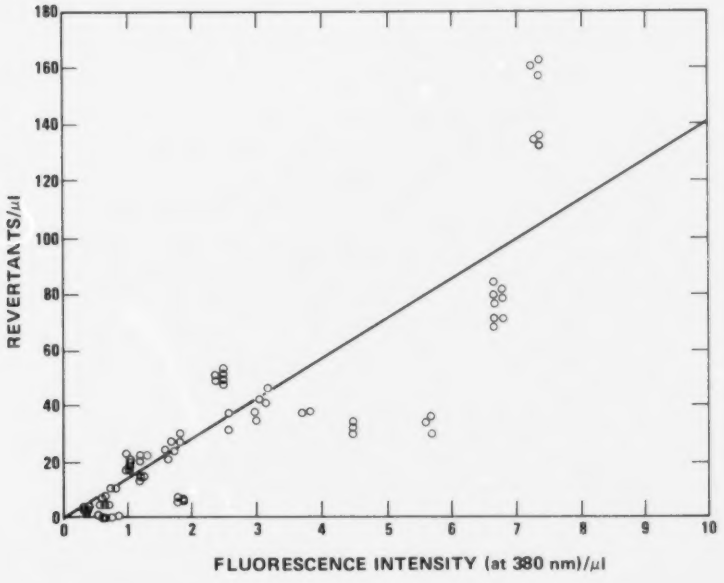


Fig. 6. Correlation of mutagenic activity of organic extracts and human serum extracts of diesel particles to fluorescence intensity at 380 nm. The correlation coefficient, P = 0.90, was significant within 99% confidence interval.

mutagenic and carcinogenic in a battery of in vitro and in vivo bioassays [Huisingh et al, in press; Nesnow and Huisingh, 1980], questions still remain regarding the bioavailability of these mutagens under physiological conditions.

This study demonstrates that incubation of diesel particles with both serum and lung cytosol removes a substantial amount (79–85%) of the solvent-extractable mutagenic activity from diesel particles. The serum associated mutagens, however, were largely non-detectable when the serum was tested in the Salmonella mutagenesis bioassay. Incubation of this serum with protease did increase the mutagenic activity of the serum and increased the binding of mutagens to XAD-2 resin. This suggests that a portion of the mutagens may be bound to serum or lung cytosol proteins, and therefore unavailable for interaction with Salmonella DNA.

It appears, therefore, that mutagens can be released from diesel particles when these particles come into contact with serum and lung cytosol. For instance, the serum and lung cytosol extracts exhibit the same excitation maximum at 340 and 400 nm as were found in the organic extracts. However, the organics excitation spectra also contained significant fluorescence at excitation wavelengths below 310 nm, which were not present in the serum or lung cytosol extracts. Apparently the organic extracts contained components having significant absorption and fluorescence at shorter, more energetic wavelengths. This would suggest that serum and lung cytosol are tending to preferentially extract larger aromatic molecules relative to organic extracts. The fluorescence spectra tend to confirm this idea.

The protease treatment did not recover all the mutagenic activity lost from the particles after treatment with serum and had only a slight effect on the lung cytosol extracts. The very weak excitation spectrum of lung cytosol would suggest substantial alteration of the extracted molecules. This finding is in agreement with studies in progress in our laboratory which indicate that lung cytosol contains components which substantially reduce the mutagenic activity of nitroaromatic compounds. Although substantial mutagenic activity is removed from diesel particles incubated with serum or lung cytosol, evidence suggests that these mutagens are bound by proteins and/or metabolized so that very little mutagenic activity is directly detectable by testing the serum or lung cytosol in the Salmonella mutagenesis bioassay. Recent studies showing that diesel particles are engulfed by mammalian cells [McCormick et al, in press; Chescheir et al, 1980] and are capable of causing gene mutations [Chescheir et al, 1980] emphasize the importance of determining the chemical nature of these mutagens and their release from particles or direct interaction with potential genetic targets.

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